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Composition and properties of the external and internal microflora of millipedes (Diplopoda)

I.M. SZABÓ, K. JÁGER, E. CONTRERAS, K. MÁRIALIGETI

Department of Microbiology
Eötvös University, H 1088 Budapest (HUNGARY)

A. DZINGOV

Faculty of Forestry
Kiril and Metodij University, Skopje (YUGOSLAVIA)

GY. BARABÁS

Biological Institute
Medical University, Debrecen (HUNGARY)

&

M. POBOZSNY

Zoosystematical and Ecological Institute
Eötvös University, Budapest (HUNGARY)

Summary :

Millipedes permanently transport large amounts of hydrophobic, mostly not ornamented (smooth) conidia of many species of *Streptomyces* adhered to the tarsal region of their legs. On the latter *Streptomyces* spp. are only passive travellers, using the animal as vector to their dissemination, and, in general, they do not occur actively either on external or in internal (gut-) habitats of the millipedes' body at all.

The fresh faecal pellets of *Ch. projectus* always contain dense populations of *Promicromonospora* (Actinomycetales) a very particular genus of up till now unknown biology and ecology, described on the basis of only some random isolates. Since their distribution is restricted only to the gut and faeces of species of Diplopoda, we suppose its possible close coevolution with millipedes as host organisms.

The millipede-faeces contain, besides *Promicromonospora*, a very complex bacterial community composed of widespread soil bacteria (bacilli, mircrococci, coryneform-like organisms, etc.) as well as diverse types and species of Enterobacteriaceae and related organisms.

Key words : Promicromonospora, Diplopoda, gut-actinomycetes.

I. Introduction

Although it is already more than 130 years ago since LEIDY (1850) discovered particular filamentous microorganisms, called *Arthromitus*, in the intestine of millipedes, our present knowledge of the gut bacterial communities of these large, litter feeding invertebrates is still very unsatisfactory. At present, it is without question that the gut of millipedes contains large, complex populations both of true but widely distributed intestinal bacteria and of common soil microbes having particular preference for the digestive canal of these animals. E.g. BALEAUX & VIVARES have demonstrated (1974) that the members of the gut-community of *Schizophyllum sabulosum* var. *rubripes* belong to at least five different genera : *Klebsiella, Corynebacterium, Sarcina, Bacillus* and *Plectridium*. Presumedly both the highly specific gut-inhabitants as well as the more or less stable, but aspecific, gut colonizers, can play a role in the intestinal metabolism, about which we know only little, although it has a great importance in soil biology. In our shortly outlined studies that follow, we have approached the problems of microbiology of millipedes in two directions :

1. Detecting the transformation of fallen leaf materials, with the aid of chemical analyses, under the influence of millipedes' feeding activities.

2. By comparing the biochemical properties and the detailed species composition of bacterial communities colonizing the fresh faecal pellets of these animals, their natural soil environments and the animals' external surface. In isolating the faeces-colonizers, we have separated isolates of diverse species of soil- and enterobacteria, furthermore those of a very characteristic, particular type of monospored, filamentous actinomycetes, which is limited in its natural occurrence with extreme preference for the millipede intestinal milieu.

II. Materials and methods

1. Sampling sites

Fallen leaves of sessile oak (*Quercus petraea* as well as adults of *Chromatoiulus projectus* were collected for feeding experiments on a site with a brown forest soil in W. Hungary. To isolate gut-, faecal-, and exoskeleton-bacteria, specimens of diffe-

rent spp. of Diplopoda were collected from the A_O- and A_F-horizons of brown forest- and rendzina-soils in W. Hungary, between 1977 and 1980.

2. Feeding experiments

Leaf feeding experiments were carried out in the zoological laboratory of the Eötvös University located in the Baradla-cave (North Hungary), with a nearly moisture-saturated atmosphere, in the dark, at a standard temperature of 10°C. Each feeding run of one month's duration was performed with five parallels in earthenware pots containing weighed specimens of *Ch. projectus*, also differently weathered air-dry leaf matter of sessile oak wetted by spraying with distilled water. Chemical analyses were performed as described by ZICSI & POBOZSNY (1977).

3. Isolation of the external microflora

Freshly collected specimens were repeatedly placed for a few minutes in Petri dishes, onto the surface of glucose ammonium-posphate-, nutrient-, starch casein- and glycerol arginine- agar plates. After removing the slowly moving animals, the plates were incubated in the dark at 28° C for 3-12 days. The colonies that appeared were then transferred, in large numbers, onto slopes of adequate agar media.

4. Isolation of bacteria from fresh faecal pellets

After removing coarse soil particles from their body-surfaces, freshly collected animals were placed onto a sterile wire screen which allowed their faeces to fall into a sterile dish below. The dropped faecal pellets, not more than a few hours old, were then suspended in water by shaking for 10 min on a reciprocating shaker, serially diluted and spread in Petri dishes onto the surface of agar-plates of different compositions (starch casein-, synthetic glucose- and nutrient-agar). Isolations were made randomly in all cases onto such agar slants, the nutrient composition of which was identical with that of the agar plate from which the isolate was picked up. Isolates were purified by repeated spreadings and reisolations, numbered as strains and maintained by continuous cultivation.

5. Identification of actinomycetes

Selected strains of *Promicromonospora* isolated by us and the type-strain of *P. citrea* (USSR RIA-562) received from the ATCC as strain No. 15908 were simultaneously studied. *Streptomyces* strains were diagnotized according to the methods and descriptive criteria used by the collaborators of the International Streptomyces Project (ISP; SZABÓ & MARTON, 1976) and identified by direct comparisons with authentic type strains and/or by using the species-redescriptions of ISP.

6. Identification of bacterial isolates

Strains of faecal bacteria were, at first, subjected to about twenty diagnostic tests and, on this basis, a part of them has been tentatively identified at generic level. After this, in order to limit the number of strains to be used in further work, representative ones were selected from among our gut strains. These latter were studied simultaneously with labeled authentic strains of more or less closely related species, which were later also involved in the comparisons. Finally, the identifications (where it was possible at all) were performed by computer aided numerical analyses, calculating similarity coefficients between pairs of strains, and clustering the similar strains on the basis of a total of 216 coded cultural-morphological and biochemical features (for some of them see Table II).

7. Cell-wall analyses

These analyses were performed on cultures grown on yeast extract starch-broth for 3 days at 28° C. Cells were harvested, washed and freeze dried. Crude cell-wall preparations were obtained by crushing cells in an X-press and centrifuging to remove conidia and intact hyphal bits. The wall-material was thoroughly washed and freeze dried. Cell-wall sugars and amino acids were determined by the methods of BARABÁS & SZABÓ (1965).

III. Results and discussion

1. The role of *Ch. projectus* in decomposition of leaf-litter

The adults of *Ch. projectus* and their microbial partners in the gut and accumulated faeces decomposed during one month more than 17 per cent of the consumed leaf litter. At the same time, less than 45 per cent of the consumed lipids and phenolic compounds disappeared, but the amount of water soluble carbohydrates increased in all feeding runs which may be explained by increasing growth of bacteria forming mucous, extracellular glycocalyx of polysaccharide nature in fresh and ageing faecal pellets.

2. Composition and role of the surface-microflora of the exoskeleton

Cells, conidia, spores and other propagules of soil microbes associated with minute organic and inorganic soil particles adhere in the largest number to the enlargements and spine — like projections of the tarsal region of legs —, and accumulate in the spaces between them. These tarsal microbial assemblages are, in general, very complex, but mostly inactive and composed chiefly of randomly collected conidia of actinomycetes from the soil. Comparing the composition of total microfloras of

TABLE I

A comparison of some *Promicromonospora* -strains isolated from the faeces of *Ch. projectus*, according to selected diagnostic features.

Features	Strain No.				Features	Strain No.			
	DFA 2	DFA 13	DFA 18/II	DFA 20		DFA 2	DFA 13	DFA 18/II	DFA 20
Branching hyphae	+	+	+	+	Growth at 2° C	+	±	+	±
Aerial mycelium	−	−	−	−	Growth at 35° C	−	−	−	−
Conidia	+	+	+	+	NaCl tolerance (> 5 %)	−	−	−	−
Breaking into coccoid element	+	+	+	+	Resistance to 80° C for 5 minutes	−	−	−	−
Gram-staining	+	+	+	+	Sensitivity to				
					Chloramphenicol	+	+	+	+
Motility	−	−	−	−	Chlortetracycline	+	+	+	+
Melanoid pigment	−	−	−	−	Streptomycin	+	+	+	+
Yellow endopigment	+	+	+	+	Oleandomycin	+	+	+	+
Voges-Proskauer	−	−	−	−	Erythromycin	+	+	+	+
Hydrolysis of Gelatin	−	−	−	−	Cephalosporin	+	+	+	+
Tween-80	−	−	−	−	Oxacillin	−	−	−	−
Casein	±	±	±	±	Methicillin	−	−	−	−
Aesculin	−	−	−	−	Colistin	−	−	−	−
Starch	±	±	±	±	Lincomycin	−	−	−	−
Cellulose	−	−	−	−	Utilization of				
Glucose breakdown (HUGH & LEIFSON-test)					Arabinose	+	−	−	+
					Xilose	+	+	+	+
					Fructose	+	+	+	+
Oxidative	+	+	+	+	Lactose	+	+	+	+
Fermentative	+	+	+	+	Melibiose	+	−	−	+
Phosphatase	+	+	+	+	Sucrose	+	+	+	+
Nitrite from nitrates	+	+	+	+	Adonitol	−	−	−	−
NH_3 from peptone	±	±	±	±	Mannitol	−	−	−	−
Phenylalanine deamination	−	−	−	−	Glycerol	+	+	+	−
Indol	−	−	−	−	Raffinose	−	−	−	−
Catalase	+	+	+	+	Dextrine	+	+	+	+
Growth with inorg. N-source	+	+	+	+					

fresh faecal pellets, litter and legs using starch casein-agar for plating, the percentage of actinomycetes proved to be 18-34, 2.3 and 95 respectively. The qualitative differences within the actinomycete fractions were, however, also enormous. The actinomycete population of the legs was nearly entirely composed of *Streptomyces* spp., while Streptomycetes occurred in the faeces only sporadically or not at all. From agar-surface prints of the legs of a few adult specimens of *Ch. projectus* collected from the $A_O + A_F$ - horizon of a brown forest soil we have isolated members of at least 17 spp. of *Streptomyces (St. xantophaeus, St. parvus, St. nasvillensis, St. nitrosporeus, St. flavidovirens, St. plicatus, St. melanosporofaciens, St. spadicis, St. galtieri, St. albogriseolus, St. umbrosus, St. badius,* etc.). This number of species is approximately equal to the highest number of *Streptomyces* spp. isolated by us, in general, from forest litter sites.

Only very few non-filamentous bacteria were isolated from the legs. The conidia of all strains of *Streptomyces* spp. isolated from leg-prints which were studied with the electron microscope showed without a single exception on ornamented smooth outer sheaths. This indicates that in the attraction of these strongly hydrophobic negatively charged propagules to the hydrophobic cuticle of millipedes the mechanical connection with the aid of spiny or hairy surface ornaments plays a less important role than the chemical nature of the spore surface (see also RUDDICK & WILLIAMS, 1972). Millipedes do not contaminate their own body surfaces with the microflora of their faeces, the members of which practically did not isolate from the legs' agar-prints.

3. *Promicromonospora* as codominant elements of the millipedes' gut community.

As mentioned before the number of actinomycetes counted on starch casein-agar may fluctuate in the dense microflora of fresh faecal pellets of *Ch. projectus* adults, between 18-34 % of the total. In contrast, they form in the microflora of the animals' litter habitat a very small fraction (1.6-3.4 %). In the litter, as well as on the legs, streptomycetes occurred nearly exclusively, but we found these in the faeces sporadically. This enormously large actinomycete-community in the droppings actually represents only one dense population of a single variable species of bacteroid appearance in smears. The first analysis of this population was performed on pure cultures of 65 selected strains. Some of them are shown in Table I. All of them proved to be able to produce branched, Gram-positive, not acid-fast substrate hyphal filaments of about 0.5-1.0 μm in diameter, breaking into often pleomorphic, mostly coccoid and short rood-shaped non motile elements of variable length. Several of them were able to form single, sessile, oval spores. They did not produce aerial mycelium and their yellow coloured old colonies became soft and easily removed from surface of medium. In the Hugh & Leifson-test they utilized glucose also under anaerobic conditions ! They have a cell-wall composition of type VI : their walls do not contain diaminopimelic acid isomers at all, but the presence in that of lysine, alanine, glutamine and

TABLE II

Cultural-morphological and physiological features of some selected strains representing individual types of faecal-bacteria of *Chromatoiulus* spp.

Features \ Strain	*Bacillus cereus* No. V/30	*Micrococcus luteus* No. V/60	Undetermined « Coryneform » (?) No. I/2	*Enterobacter (aerogenes)* No. X/86
Cells	Rods	Spheres	(Rods)	Rods
Dimension μm	1.0 by 2.3–3	1.0–2.0	0.8 by 2–12	0.8 by 2.0
Gram-staining	+	+	+	−
Motility	+	−	−	+
Spores (C = central; E = elliptical)	+ (C;E)	−	−	−
Pigmentation	White	Yellow	Light reddish	White
Indole produced	−	−	−	−
NO_3 to NO_2	+	−	−	+
Catalase	+	+	+	+
Cit. oxidase	−	−	−	−
Acetoin	(−)	−	−	+
Phosphatase	+	+	+	+
Urease	−	−	+	−
H_2S	−	−	−	−
Growth at 37° C	+	+	+	+
Hugh & Leifsen O/F test	Ferm.	Ox.	Ox.	Ferm.
Lactose fermentation	−	−	−	+
Lecithinase	+	−	−	−
Growth in 5 % NaCl	+	+	+	+
Methyl red	+	−	−	−
Citrate (Simmons)	−	−	+	+
Methylene blue red.	+	−	−	+
Phenylalanine deamination	−	+	+	?
Ammonium salts as sole N-source	±	±	+	+
Hydrolysis of Gelatin	+	?	−	−
Starch	+	+	−	±
Esculin	+	−	±	+
Casein	+	+	−	−
Hippurate	−	−	+	+
Tween-80	+	+	−	+
Arginine	−	−	−	+
Acid produced from Glucose	+	±	+	+
Arabinose	−	−	−	+
Xilose	−	−	−	+
Mannitol	−	−	+	+
Sensitivity to Erythromycin	+	+	+	±
Streptomycin	+	+	+	±
Tetracycline	+	+	+	±
Penicillin	−	+	+	−
Oxacillin	−	+	−	−
Lysozyme	+	−	+	+

threonine was detected in 1:1:1.08:0.3 mol ratio. In wall preparations, aspartic acid, serine, proline, glycine, cysteine, methionine, leucine, isoleucine, phenylalanine, tyrosine, histidine and arginine were completely lacking. On the other hand, while the presence of rhamnose, mannose, fucose and glucosamine was also detected, glucose, galactose, galactosamine, ribose, arabinose and xylose proved not to be cell-wall constituents. This particular constellation of morphological, physiological and chemical properties makes it possible to place our gut actinomycete strains into the genus *Promicromonospora* (LECHEVALIER & LECHEVALIER, 1981), showing considerable similarity but not identity with the type strain (ATCC 15908) of *P. citrea*. These findings throw light on the true natural habitat of this very particular, less known group of bacteria, and thus it will be possible to investigate the ecological biochemical and taxonomic problems of them in much larger strain-materials than earlier. The common occurrence and dense growth of *Promicromonospora* is characteristic of the gut milieu of many species of millipedes. We have isolated and are now studying hundreds of strains belonging to this group of organisms also from the faeces of *Leptoiulus proximus, Cylindroiulus luridus, C. boleti* and *Unciger foetidus*.

4. Composition of the bacterial population in the fresh faecal pellets

From 165 and 79 isolates obtained from fresh faecal pellets of *Ch. projectus* and *Ch. boleti*, respectively, we have separated on the basis of the first tentative groupings altogether 91 representative strains which, together with 30 authentic strains of related species, were involved in further detailed, comparative diagnostic studies. The computer — using 216 coded features — created from them about 18 separate clusters of similar strains. These similarity groups which were taxonomically still not in each individual case identified, and about which we will report in detail elsewhere, may be considered as units or taxa partly at generic and partly at specific level. Taking the obtained results into consideration, it may be stated first, that the faecal-flora of millipedes is very complex, composed of more species and genera as we thought earlier. Secondly, the statement of BALEAUX & VIVARES (1974) that the gut flora of *Schizophyllum sabulosum* (Diplopoda) is composed partly of soil, partly of true intestinal bacteria seems to be valid also for *Chromatoiulus* spp.; e.g. soil bacilli and micrococci frequently may occur as characteristic but neither qualitatively nor quantitatively constant or stable members of microbial populations in *Chromatoiulus* droppings. From the faecal matter of *Ch. projectus* 18 strains of *Micrococcus luteus* were isolated. Selected properties of one of them (strain No. V/60) are presented in Table II. A strain of *Bacillus cereus* (No. V/30) from among the 29 isolated from the faeces of *Ch. boleti* is also shown in Table II. These descriptions, involving the key-characters of the species in question too, confirm the correctness of their identification. From the droppings of *Ch. projectus*, we did not isolate bacilli. Presumedly the occurrence of the latter in the gut depends strongly on unknown external and internal environmental factors. The density of *M. luteus* population in droppings of

Ch. projectus we found to be large enough to let us suppose the active participation of these organisms in intestinal processes. The problem of the occurrence of coryneform bacteria or even that of the genus *Corynebacterium* in the gut of invertebrates, among them Diplopoda, is still more confusing although some authors consider these organisms true gut inhabitants of arthropods. In smears, among others, the cells of *Promicromonospora* resemble coryneforms and e.g. the *Arthrobacter* sp. of JONES & BALLOU has been found to be an « NMO » by LECHEVALIER (1972). In Table II, we present also an undetermined « coryneform »-strain (No.I/2) which was selected by us from among 27 similar or identical strains isolated from *Ch. boleti* casts. The main problems which arose at the taxonomic level were in recognizing those strains which proved to be the members of family Enterobacteriaceae. A lot of intermediate types and variants of them and of related microbes were isolated by us. From among these, one *Enterobacter* strain (No. X/86) isolated from the faeces of *Ch. projectus* is presented in Table II. Strain No. X/86 seems to be an atypical member of *Enterobacter aerogenes*, a species which was found also among the gut inhabitants of *Bibio marci* (Diptera) larvae (SZABÓ, 1974). At present we are continuing the taxonomic work with them.

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Discussion

GHILAROV, M. : From where do the microorganisms get into the gut of millipedes since they seem to be absent in the soil and in the litter ?

SWIFT, M.J. : Can you tell us how the microbes are transmitted between animal generations ?

SZABÓ, I.M. : The *Micromonospora* occur only in traces in the soil-habitat of millipedes. A few cells might be enough to infect these animals. It is also possible that millipedes have a particular transfer mechanism.

MEYER, E. : Could you tell us if these millipedes are dependent on the presence of *Promicromonospora* in order to obtain nutrients from the leaves ?

SZABÓ, J.M. : The millipedes' dependence on *Promicromonospora* is always an open question. Strains of *Promicromonospora* can grow on artificial substrates in the laboratory, and under such isolated circumstances they can be cultivated indefinitely.

JAGNOW, G. : What happens to the *Promicromonospora* in the faeces after leaving the intestinal gut ? Do they rapidly die ?

SZABÓ, I.M. : During the microbial succession in ageing faecal pellets the *Promicromonospora* disappear relatively quickly, and it occurs in the soil only sporadically or not at all. The reason perhaps is that the members of this genus are too one-sidely adapted to the intestinal milieu.

ZINKLER, D. : The *Promicromonospora* colonize densely the hindgut of millipedes. Does the hindgut work as a fermentation chamber ? Does the absorption of degraded components take place into the millipedes ? Does there exist a special epithelium ? Is it possible to label the millipede food with ^{14}C ?

SZABÓ, I.M. : The *Promicromonospora* strains can ferment glucose under laboratory conditions. Presumably the *Promicromonospora* are also taking part in the fermentation processes of the gut. It is questionable that the hindgut of millipedes functions as a fermentation chamber, but it seems to be sure that in the hindgut occur many anaerobic microsites which favour anaerobic lysis. For the present we have only few data in the literature. About the functional anatomy of the gut of millepedes the labelling of millepede food with ^{14}C is hardly possible.

GHILAROV, M. : Is the increase of N-content in millipede faeces partly connected with the extraction of uric acid into the hindgut through the Malpighian tubules ?

SZABÓ, I.M. : The N-content of dropped faecal pellets (not older than 1 month) was measured. The increase in N-content may be due, partly to the excretion of N-containing compounds by the animal, but chiefly to the bacterial N_2-fixation in the gut and in the ageing faecal pellets. The N-metabolism of this millipede-bacterial system needs further more detailed investigations.

DOMSCH, K. : An increase in the absolute N-content of pellets was observed in the course of two months. Has the pellet weight been corrected for loss of organic matter and has the N_2-fixation actually been measured ?

SZABÓ, I.M. : The change in N-content was calculated by comparing the amounts of N in the consumed litter and in the produced faeces. The N_2 -fixation by bacteria has actually not been measured. The weak increase in absolute N-content in June (6 %) is not typical. The large increase (31 %) in October cannot be explained only by the bacterial N_2- fixation. We suppose that in this feeding run the animals have metabolic troubles and secrete more N-compounds than they assimilate; consequently, the overwhelming majority of the consumed litter is utilized by the microbial partners.

ANDERSON, J.M. : We have also observed *Micromonospora* - like filamentous bacteria in the hindguts of many other millepedes, particularly julids (though they are not a conspicuous feature of *Glomeris*). Do you think that they are functionally important for the animals and would you predict any effects of this species dominance on other gut microflora (for example Trichomycetes) ?

SZABÓ, I.M. : We may suppose that the co-dominant occurrence of *Promicromonospora* in the millipede gut is associated with important community functions. They might influence the activities of many other members of the gut population (but the way in which these influences are working is not clear). The majority of the strains did not produce antibiotic substances and showed good growth with inorganic N-sources. This is a property of outstanding ecological importance in the course of natural selection.

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